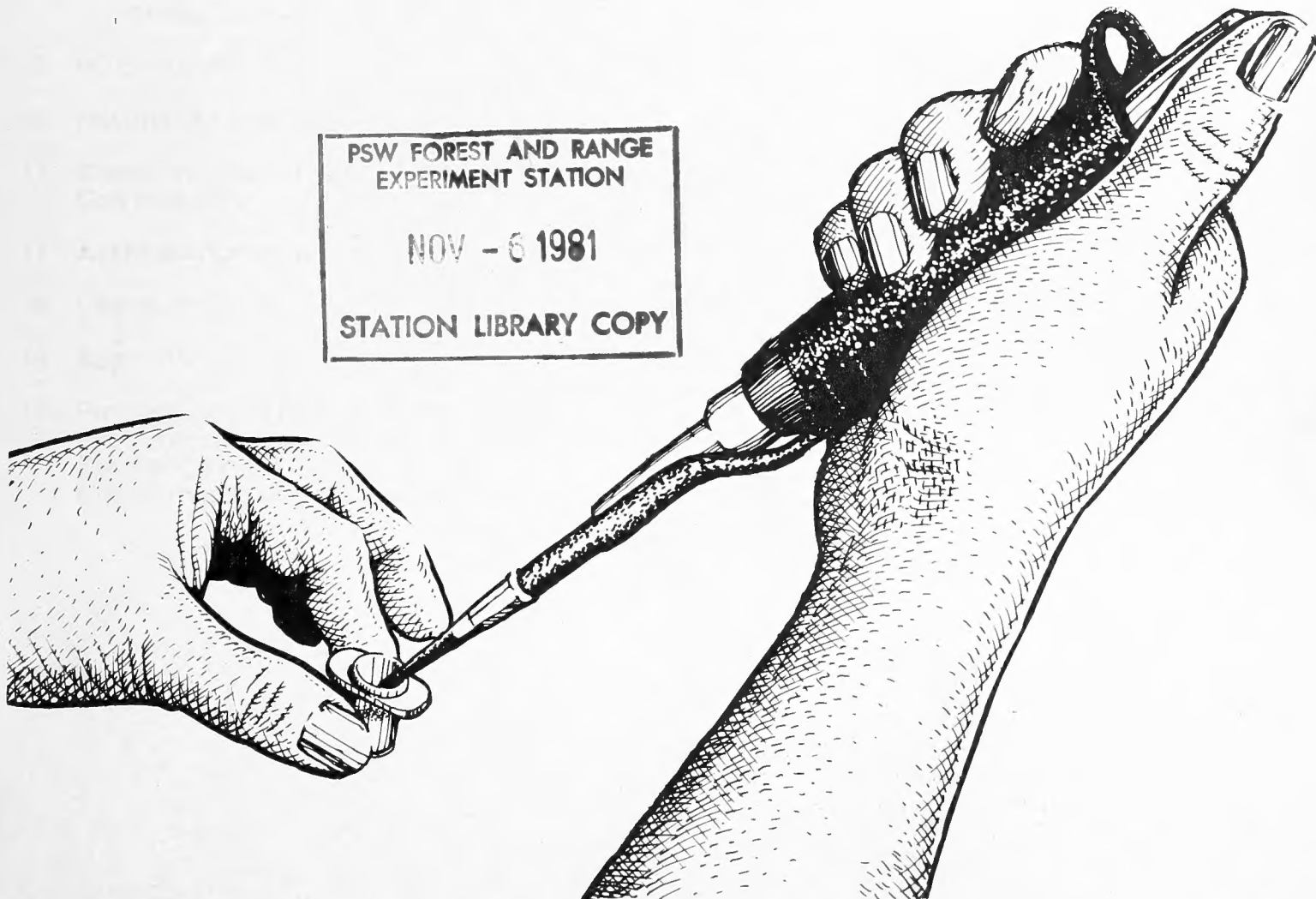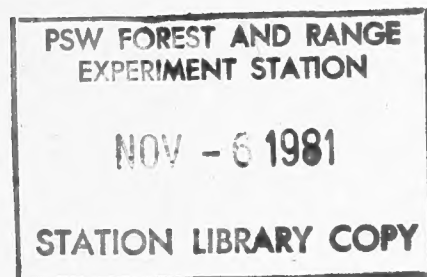# Historic, archived document

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Peroral Bioassay of Nucleopolyhedrosis Viruses in Larvae of the Western Spruce Budworm

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Contents

Abstract

The relative virulence of entomopathogenic viruses and the potency of virus preparations for control of destructive insects can be estimated reliably only by means of biological assay in the target species. A simple, yet sensitive peroral bioassay procedure is described for preparations of nucleopolyhedrosis viruses pathogenic for the western spruce budworm, *Choristoneura occidentalis* Freeman. This procedure has been used to compare the activity of *Baculovirus* isolates from several conifer-feeding species of *Choristoneura*.

Summary

No information is available on the activity titers of viruses pathogenic for the western spruce budworm, *Choristoneura occidentalis* Freeman. Quantification of the biological activity of these viruses is needed, both for laboratory investigations and for field tests against this destructive defoliator. Baculoviruses isolated from nearctic and palearctic conifer-feeding species of *Choristoneura* are generally cross infectious. Thus, a bioassay method is essential for comparing the virulence of virus isolates in a screening program of pathogens of the western spruce budworm. The method described here minimizes the difficulties usually encountered in bioassays and standardizes the various operations needed for measuring the activity of pathogens in a fastidious host. The procedure—although relatively simple—has a high degree of reproducibility, and it does not require instrumentation other than that available in most biological laboratories. The precision of the assays compares favorably with that known from other virus-host systems.

Data from three bioassays and a statistical comparison of dose-mortality regression lines illustrate an application of the peroral bioassay method in virus screening. An appendix describes simplified methods for preparing experimental quantities of baculoviruses (0.5-2 g of inclusion bodies) suitable for biological assay and biochemical studies.

Future field applications of viruses against the western spruce budworm should be based on the polyhedron-to-bioactivity ratio of each pathogen rather than on polyhedron counts only. This bioassay procedure offers a method of activity titration for estimating field doses of baculovirus preparations, as generally recommended in tests with microbial control agents, and as required specifically by U.S. pesticide regulations.

Introduction

The western spruce budworm, *Choristoneura occidentalis* Freeman, is one of the most destructive defoliators in western North America. This insect occurs principally on true firs and Douglas-fir in the Pacific Coast States, British Columbia, and the Rocky Mountain States (Carolin and Honing 1972, Furniss and Carolin 1977). Although modern forest-insect management frequently includes the use of microbial insecticides, surprisingly little is known about the diseases of *C. occidentalis* or their role as biological control factors. Among the viral agents, nucleopolyhedrosis virus (NPV), *Baculovirus* subgroup A, has been isolated from wild populations of this insect in the Bitterroot Range of Idaho, in 1967, and in the Blackfoot River drainage near Missoula, Montana, in 1969 (Thomas and Poinar 1973). Unfortunately, these isolates were not preserved (G. M. Thomas, Division of Entomology and Parasitology, University of California, Berkeley, California 94720, letter on file, Forestry Sciences Laboratory, Corvallis, Oregon 97331, personal communication). In 1976, NPV was isolated from *C. occidentalis* in Canada (Cunningham et al. 1978). This virus, after passage in *C. fumiferana* (Clemens), was used in field tests in British Columbia to control *C. occidentalis* (Cunningham et al. 1978). Populations of *C. occidentalis* were also treated with NPV isolated from *C. fumiferana,* with encouraging results in 1978 tests (Cunningham and Percy 1981). In 1979, NPV was isolated in our laboratory from larvae of *C. occidentalis* collected at White Sulphur Springs, Montana. The larvae were obtained in July from G. P. Markin, Davis, California (C. G. Thompson, Forestry Sciences Laboratory, Corvallis, Oregon 97331, personal communication).

On the other hand, a vast amount of information is available on the pathology and epizootiology of nucleopolyhedrosis and other viral diseases of *C. fumiferana,* the spruce budworm common in eastern North America (Cunningham 1978; Cunningham and Percy 1981). Since 1971, over 2000 ha of forest in Canada were sprayed with NPV. Aerial applications of this virus reduced populations of *C. fumiferana,* with variable success, from less than 20 percent to more than 90 percent. Satisfactory foliage protection was achieved in some of the treatments (Cunningham and Percy 1981).

Unfortunately, no information is available on the potency of viral preparations used in laboratory investigations or in field tests against both species of spruce budworms. No attempts to standardize the activity of spruce budworm NPV have been reported. This is most likely because propagation of spruce budworm viruses and their field use have been on a modest scale only, and no current commercial interest has been shown in developing a viral insecticide for control of these insects. Procedures for activity titration and activity standardization[1] of NPV are useful not only for comparative potency assessments of products used in field trials but also for comparison of virus strains and isolates (Martignoni and Ignoffo 1980). In addition, the U.S. Environmental Protection Agency (1975) requires that quantification of nucleopolyhedrosis and granulosis virus products be based on bioassays rather than counts of inclusion bodies. Baculoviruses found in nearctic conifer-feeding species of *Choristoneura* and in the European *C. murinana* (Hübner) are generally cross infectious (Stairs 1960; Cunningham and Percy 1981; M. E. Martignoni, unpublished tests); methods for comparing the virulence of these diverse viruses in vivo were needed.

In 1978, we started a screening and evaluation program for NPV pathogenic for larvae of the western spruce budworm. Several of these viruses are now considered good candidates for field testing against *C. occidentalis.* The bioassay procedure described here was developed over the past 2 years, and it was tested repeatedly in its present form. This procedure is based in part on a bioassay method for baculoviruses of the Douglas-fir tussock moth, *Orgyia pseudotsugata* (McDunnough), (Martignoni and Iwai 1977) and is similar in principle to that used by Ignoffo (1966) for NPV of *Heliothis zea* (Boddie). The bioassay method for NPV of *O. pseudotsugata* was approved as part of the quality-control procedures of the virus product designated "TM BioControl-1" (EPA Registration No. 27586-1, August 11, 1976).

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[1]The determination of activity of a virus, or biological assay, is a process by which the relation between virus dose and response in susceptible hosts is tested. The activity titer or potency of a virus preparation is the amount of activity per unit weight or volume of the preparation, measured in activity units. Standardization of activity is a process by which the activity of a virus is measured in standard activity units, units accepted by consent as a basis for comparison. (Standardization can be of limited import, e.g., within one laboratory or among few laboratories, or it can extend nationally and internationally as it does for *Bacillus thuringiensis* Berliner preparations.)

The procedure (summarized in table 1) is relatively simple, yet sensitive; it does not require instrumentation other than that available in most biological laboratories. We recommend it for comparison of the activity and virulence of viruses pathogenic for *C. occidentalis* and for measurement of the potency of viral preparations for field application.

Table 1—Parameters of the bioassay procedure for *Baculovirus* isolates pathogenic for *Choristoneura occidentalis*

Test insect	*Choristoneura occidentalis,* strain COC-FS-01, initiated at the Forestry Sciences Laboratory, Corvallis, in 1979
Age and sex	Post-diapause larvae, fourth instars (4-24 h after molt), fed, both sexes
Weight (mean, SD)	2.6 ± 0.2 mg
Confinement	One larva per standard 2-ml analyzer cup with perforated cap
Diet and volume	Modified McMorran diet, 1 ml per cup
Rearing temperature	$30° \pm 0.5°C$
Light regimen	Continuous darkness during holding period
Holding period	14 days
Exposure to the virus	Diet-surface treatment, larvae continuously exposed
Inoculum volume	25 μl per cup (diet surface area: $109 \, mm^2$)
Group size	30 larvae per group, at start; nonfeeding larvae removed at 48 h
Replication	Two groups (60 larvae) per dose level
Dose levels	Four
Serial dilution factor	1/3
Control (untreated)	One group (30 larvae)
Total number of larvae	270 per assay
Observations	Periodically, record number of dead larvae; record number of living larvae on the 14th day; determine the presence of nuclear inclusion bodies by microscopic examination of tissues of dead larvae
Evaluation	Maximum-likelihood logistic regression analysis (LC_{50}, LC_{90}, LC_{99}, confidence limits, precision, and slope)
Units	Nanograms (dry weight) of virus powder per cup; picoliters of stock virus suspension per cup; polyhedral inclusion bodies per cup

Rearing and Selection of Insects for Tests

I. Maintenance of *Choristoneura occidentalis* Stocks

The insect-rearing procedures and diet formulation were essentially as described by Lyon et al. (1972) and Robertson (1979). We used routinely the diapausing colony of strain FS-01 of *C. occidentalis,* one of the insect strains maintained at our laboratory and currently in the 14th generation. The modified McMorran diet was formulated and prepared as described by Robertson (1979), with the following exceptions: wheat embryo was replaced with an equal amount of wheat germ; linolenic acid was omitted; formalin was omitted; and penicillin G was added (126 mg/l).

For bioassays, we found that the diapausing colony of *C. occidentalis* provides a very convenient supply of even-aged larvae with just 1-week's notice. Continuous rearing (as required for the nondiapause colony) is not necessary, because diapausing larvae remain viable for several months under refrigeration. With careful budgeting of the numbers of larvae needed for bioassays, mating and oviposition can be limited to three times yearly. Second instars, within their hibernacula, are stored at 2-4°C for 100-250 days, as described by Robertson (1979). After removal from cold storage and incubation at 25°C, diapausing larvae reach the fourth stage in 6-8 days.

One week before the assay begins, we remove from the cooler several petri dishes with a total of about 800 diapausing larvae per bioassay. A small cube of diet is placed in each dish, the dishes transferred to a 25°C incubator, and routine care is provided as described by Robertson (1979). For ease of handling, on the 2d and 3d days of incubation the diapause gauze pad is moved to a second and third set of sterile petri dishes (50 x 9 mm, with tight lid) with fresh diet. Eventually, about 60-70 larvae will be in each petri dish.

II. Selection of Fourth Instars for Bioassay

A. Materials and equipment[2]

1. A supply of late third instars of *C. occidentalis.* Usually, about three times the number of larvae needed for bioassay is sufficient (800 third instars for one bioassay).

2. Sterile disposable petri dishes, 60 x 15 mm, without diet (9).

3. Sterile brushes #00 (4).

4. Incubator, 25°C.

B. Procedure

1. The day before the test: At 4:00 p.m., remove the petri dishes from the 25°C incubator; find and discard all fourth instars; return the dishes to the incubator.

2. The day of the test: At 9:00 a.m., remove the dishes from the 25°C incubator; collect the required number of fourth instars from the dishes screened the previous day; place 30 larvae in each of nine sterile petri dishes, without diet (these larvae molted to the fourth stage during the preceding 17 h; their average weight is 2.6 mg, with a standard deviation of ±0.2 mg).

Note: A complete bioassay requires 30 larvae per group, two groups per dose level, and four dose levels, as well as one control group. Thus, 270 fourth instars are needed for one bioassay.

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[2]Unless otherwise specified, the amounts listed in these instructions are for one bioassay only (270 larvae). For more than one assay, increase the amounts accordingly.

Peroral Bioassay

I. Preparation of Test Cups

A. Materials and equipment

1. A supply of freshly mixed, warm (60°C) liquid diet (minimum volume: 350 ml, sufficient for 270 cups).

2. Disposable plastic Luer-Lok syringe, 10-ml, sterile, with a sterile 13G x 50-mm hypodermic needle.

3. Disposable analyzer cups, 2-ml, flat bottom, with write-on wings (Sherwood Medical Industries,[3] Lancer Division, St. Louis, MO 63103, catal. No. 11405) (270).

4. Trays, polycarbonate, with 30 holes, 14-mm diameter (9) (two such trays can be obtained conveniently by disassembly of a Nalgene polycarbonate test-tube rack, Nalge Company, Rochester, NY 14602, catal. No. 5929-0013).

5. Racks for the polycarbonate trays (2). We use a Lucite rack capable of holding six trays (fig. 1). (The construction diagram can be obtained from the authors.)

6. Sterile paper tissues (box).

7. Plastic bags (20 X 30 x 55 cm)

8. Adhesive labels (4).

9. Thermometer (for 60°C water bath).

10. Hot plate, with water bath (60°C).

11. Sterile hood.

12. Ultraviolet sterilizer.

13. Refrigerator.

NOTE: The diameter of the analyzer cup at the 1-ml level is 11.8 mm and the surface area of 1 ml of diet is 109.4 mm^2.

Figure 1.—Polycarbonate tray, with 30 analyzer cups, in place in a Lucite rack capable of holding 6 trays.

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[3]The use of trade, firm, or corporation names in this report is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the U.S. Department of Agriculture of any product or service to the exclusion of others that may be suitable.
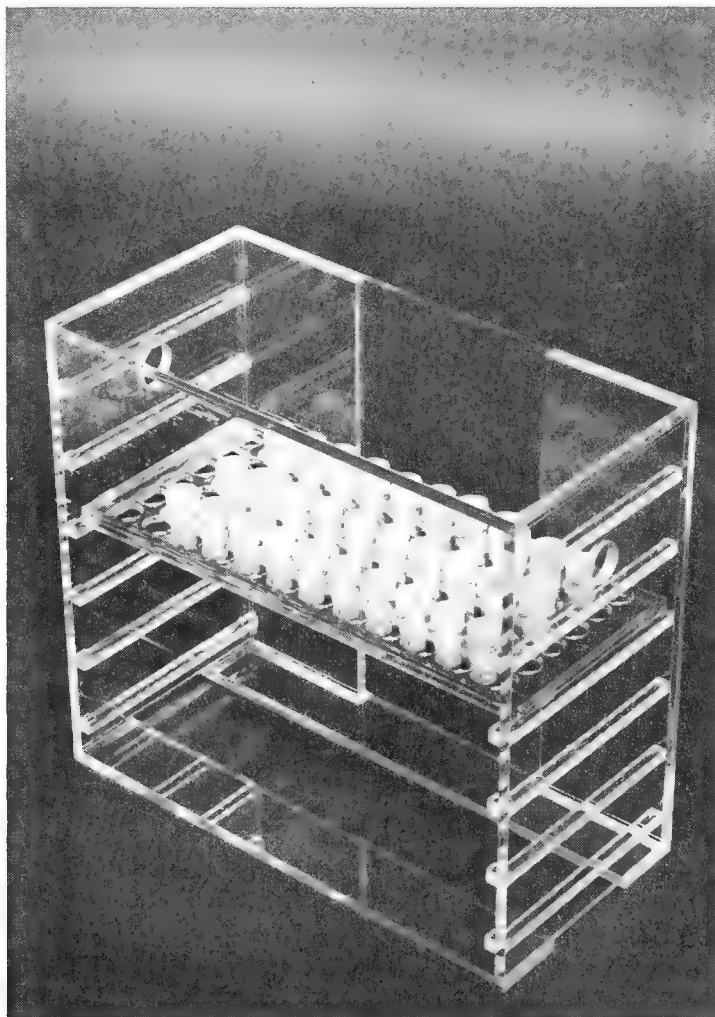
B. Procedure

1. Place 30 analyzer cups in each of nine trays. Place the trays in the UV sterilizer and expose for 30 min.

2. Place the container with freshly mixed, warm liquid diet in the water bath, at 60°C. Fill the 10-ml syringe with diet. With a sterile paper tissue, wipe the excess diet off the exterior of the hypodermic needle.

3. Place the point of the needle on the bottom of the analyzer cup, without touching the wall of the cup. Dispense 1 ml of diet. Be precise. Continue until all 270 cups have received 1 ml of diet.

4. Discard all analyzer cups with air bubbles on the surface of the diet or with streaks of diet on the wall. Replace with flawless cups.

5. Air dry the cups under a sterile hood for 1 h. The cups are now ready for inoculation, or they may be stored for later use. For storage, place the trays in plastic bags, seal, and affix a label (diet code and date dispensed). Refrigerate at 4°C. Preferably, these cups should be used within 1 week, but they can be stored and used for up to 2 weeks.

NOTE: For filling larger batches of test cups (more than three sets of 270 cups) we recommend an automatic dispensing device.

II. Preparation of Serial Dilutions of Virus

A. Materials and equipment

1. Stock virus suspension. If the virus preparation is a powder or a paste, prepare a stock suspension in saline (e.g., 50 mg in 50 ml sterile buffered saline).

2. Sterile buffered saline (100 ml), prepared as follows:

4.266 g BES [N, N-bis-(2-hydroxyethyl)-2-aminoethane-sulfonic acid], molarity 0.02;
9.000 g NaCl;
0.0075 g DOSS (dioctyl sulfosuccinate, sodium) **or** 100 μl of a 7.5-percent DOSS solution such as Laboratory Aerosol® OT (Fisher Scientific Company, Pittsburgh, PA 15219);
distilled water, about 900 ml;
adjust the pH to 7.13, at 20°C, with normal NaOH;
distilled water to 1000 ml;
dissolve, with continuous stirring, 500 mg hydroxyethyl cellulose (Cellosize QP-4400, Union Carbide Corporation, New York, NY 10017);
autoclave (in 100-ml aliquots); the pH will be 7.15 after autoclaving.

3. Disposable sterile pipettes, 1-ml and 5-ml (12 each).

4. Rubber pipette filler.

5. Adhesive labels (8).

6. Glass vials with plastic snap caps, 16-ml, sterile (8) (Wheaton Scientific, Millville, NJ 08332, catal. No. 225536); the snap caps can be sterilized by 30-min exposure to ultraviolet radiation.

7. Disposable capillary tubes, 1.5 x 75 mm (12), with rubber bulb assembly.

8. Bright-line counting chamber (improved Neubauer ruling).

9. Counter, one-unit, table model.

10. Ultraviolet sterilizer.

11. Microscope, preferably phase-contrast (16X objective, 25X eyepieces, long working-distance condenser).

B₁. Procedure for range-finding assay.—If an approximate estimate of the activity titer of a virus preparation is not available, a preliminary (or range-finding) assay is necessary. To prepare an adequate dilution series for bioassay, first obtain an approximate estimate of the activity titer of the virus preparation. If an NPV preparation is not too old and if it has not been exposed to physical and chemical inactivating agents, the number of polyhedral inclusion bodies (PIB) per unit weight or volume of the preparation is usually a rough indicator of its potency. A PIB count offers a basis for preparing serial dilutions for a range-finding bioassay. The dilution series must include the presumed extreme LC_{50} levels. As a general rule, from results of peroral bioassays of several virus isolates in larvae of *C. occidentalis,* the LC_{50} of the most virulent NPV is about 50 PIB per cup, and the LC_{50} of the least virulent isolates can be as high as 50,000 PIB per cup.

1. Dilute the stock suspension in buffered saline until a suspension is obtained that is just faintly turbid. Record the dilution steps and compute the dilution factor.

2. With capillary tubes, fill the counting areas of the counting chamber with the dilute suspension, in accordance with routine blood-cell counting procedures. Place the counting chamber on the stage of the microscope and focus with a 16X objective. The PIB are allowed to settle for 8 min. Count the PIB in the four corner 1-mm squares and in the central square and tally with a counter. If the number of PIB is high (50 per mm square is the recommended maximum), a fresh suspension of greater dilution should be prepared and the count repeated. After completing one satisfactory count in one counting area of the chamber, count the PIB in the four corner 1-mm squares and in the central square of the second counting area. The difference between the two counts in both areas of one counting chamber should not be greater than 10 percent of the mean of the two counts. Sets of two counts with a difference greater than 10 percent of the mean are discarded, and the counts repeated in a clean chamber (Martignoni 1978).

3. Compute the number of PIB in the stock suspension as follows:

$$\text{Number of PIB per microliter of stock suspension} = \frac{\text{Total number of PIB counted} \times \text{Dilution} \times 10}{\text{Number of 1-mm squares counted}}$$

4. Prepare four serial dilutions of the stock suspension, in steps of one-tenth, in sterile buffered saline; 3-5 ml per glass vial are sufficient (cover each vial with a sterile plastic snap cap). The following dose levels are recommended for a range-finding assay:

PIB per cup (25 μl per cup)	PIB per μl	Vials
50,000	2,000	1
5,000	200	1
500	20	1
50	2	1

B₂. Procedure for a narrow-range assay.—This procedure should be used if an approximate estimate of the infectivity titer of the virus preparation is available, or if a preliminary LC_{50} has been determined by means of a range-finding assay (B₁, above).

1. Prepare two replicate sets of four serial dilutions of the stock suspension, in steps of one-third, in sterile buffered saline; 3-5 ml per glass vial are sufficient (cover each vial with a sterile plastic snap cap). These dilutions must be computed so that two of the dose levels will be above and two below the preliminary or estimated LC_{50}.

Example:

From the data gathered in a range-finding assay, we calculate that the LC_{50} is about 260 PIB per cup. The following dose levels will then be considered appropriate for a narrow-range assay:

PIB per cup (25 μl per cup)	PIB per μl	Vials
1,350	54	2
450	18	2
150	6	2
50	2	2

Note: The dilute virus suspensions, for narrow-range as well as range-finding assay, must be prepared shortly before inoculation of the test cups (never earlier than 1 h before inoculation).

III. Inoculation of Cups and Transfer of Test Larvae

A. Materials and equipment

1. Analyzer cups with diet (270) (fig. 2), in polycarbonate trays (9) (see I, this section).

2. Racks for polycarbonate trays (2).

3. Perforated caps, for analyzer cups (270) (polyethylene beaker cap, Scientific Products, McGaw Park, IL 60085, catal. No. B2716-1). The caps are perforated with a cold needle (three holes, approximately 0.3 mm diameter), washed, rinsed in distilled water, dried, placed in plastic petri dishes, and exposed in a UV sterilizer for 30 min (fig. 2).

4. Fourth instars of *C. occidentalis* (270) (see II, "Rearing and Selection of Insects for Tests").

5. Dilute virus suspensions (see II, this section).

6. Sterile toothpicks (box).

7. Infectivity test record charts, p. 1 and p. 2 (figs. 3 and 4).

8. Fine-tip felt marker, permanent ink.

9. Sterile magnetic stirring bars, Teflon encapsulated, micro, 3 x 10 mm (8).

10. 25-μl pipettor, tip-ejector type, with sterile plastic tips (16).

11. Magnetic stirrer.

12. Ultraviolet sterilizer.

13. 30°C incubator.

Note: A range-finding assay requires only 30 cups and 30 larvae per dose level (no replicates); the total, with one control group, is 150 cups and 150 larvae.

B. Procedure

1. Two h before inoculation: remove the trays with analyzer cups from the refrigerator, unwrap, and place them in a sterile hood, at room temperature, to eliminate condensation droplets.

2. While the cups are in the sterile hood, inscribe the test number and the group numbers on the cup wings; use a fine-tip felt marker.

3. At inoculation time (2 h after the cups have been removed from the refrigerator), place a sterile micro stirring bar in one of the vials with the most dilute virus suspension. Place the vial on the magnetic stirrer and activate the stirrer.

Figure 2.—Analyzer cup with 1 ml of modified McMorran diet; the three holes (diameter 0.3 mm) are visible in the top of the cap.

FSL Corvallis (Martignoni) 75-SBW	EVALUATE	COMPLETED	SUMMARY	COMPUTER	836

Purpose _____

Species _____ Stage, instar, mg _____

Source of insects _____ Card # _____ Gener. _____ Temp. _____ Diet _____

Inoculum _____ Volume _____ Route _____

Other data   Container

Group No.	Date treated	Inoculum	Group size at 48 h	Dead larvae (days postinoculation)										L
				3-5	6	7	8	9	10	11	12	13	14	
1				-										
				P →										
														-
2				-										
				P →										
														-
3				-										
				P →										
														-
4				-										
				P →										
														-
5				-										
				P →										
														-

P = pupated   L = live at completion

					836

Figure 3.—Record chart for bioassays (first page).

FSL Corvallis (Martignoni)
76-SBW

Continuation sheet

Group No.	Date treated	Inoculum	Group size at 48 h	Dead larvae (days postinoculation)										L
				3-5	6	7	8	9	10	11	12	13	14	
				–										
				P →										
														–
				–										
				P →										
														–
				–										
				P →										
														–
				–										
				P →										
														–
				–										
				P →										
														–

P = pupated   L = live at completion

Figure 4.—Record chart for bioassays (continuation page).

4. Insert a sterile plastic tip on the pipettor and dispense 25 μl of virus suspension in the first cup of a tray (fig. 5); swirl the inoculum in the cup, making certain that the entire diet surface is wetted.

5. Proceed to dispense 25 μl to each of 14 additional cups; swirl the inoculum in each cup.

6. Eject the pipette tip and insert a sterile tip on the pipettor.

7. Continue until all 30 cups of one tray have been inoculated; eject the pipette tip; place the tray in a sterile hood (no light), at room temperature, for a minimum of 1½ h or until the diet surface is dry.

8. Insert a sterile tip on the pipettor.

9. Repeat the above steps (3 to 8) with each of the other virus suspensions, in order of increasing concentration. Change the pipette tip after each series of 15 cups.

10. When the diet surface is dry (about 1½ h after inoculation), transfer, with a sterile toothpick, one fourth instar to each cup and cap the cups with perforated lids (fig. 6).

11. Place the trays in the racks (fig. 7) and place the racks in a 30°C incubator, for 14 days, in darkness.

12. Number an infectivity test record chart (and continuation sheet); complete the top five lines and the columns "Date treated" and "Inoculum."

Figure 5.—A tip-ejector pipettor is used for placing 25 μl of a dilute virus suspension on the diet surface, in an analyzer cup.

Figure 6.—Capped analyzer
cup with diet and a second-
stage (2.6-mg) *Choristoneura
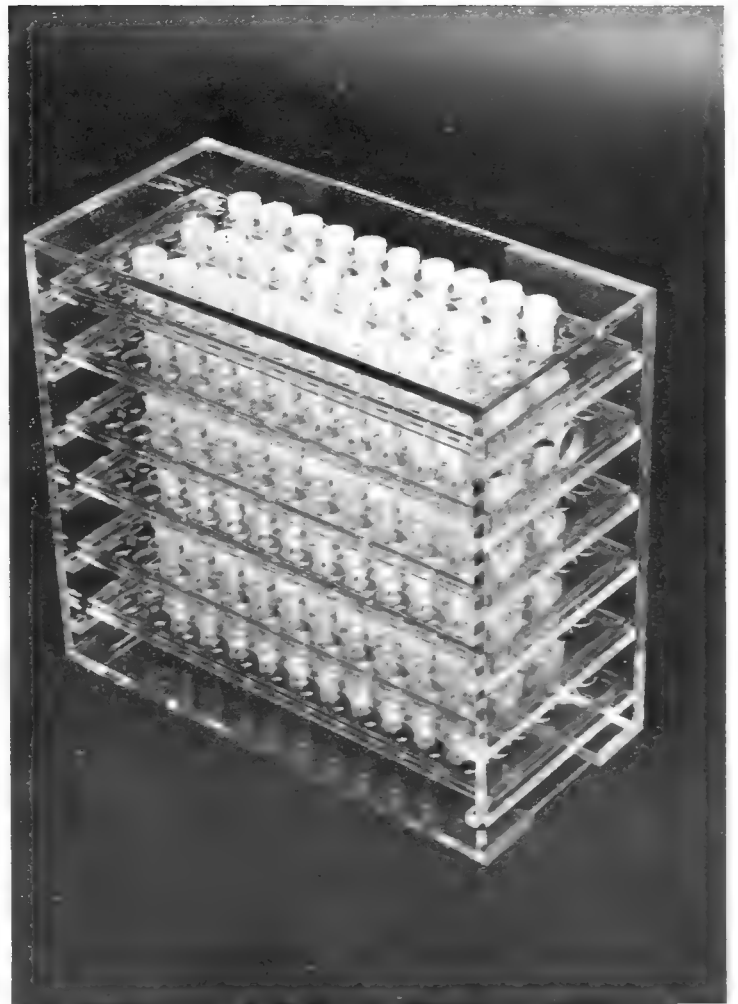occidentalis* larva.

Figure 7.—Lucite rack with 6
polycarbonate trays and 180
inoculated analyzer cups (one
second instar per cup).

IV. Evaluation

A. Materials and equipment

1. Microscope slides and cover glasses (1 box each).

2. Sterile toothpicks or sterile disposable blood lancets (box).

3. Buffered saline (see II, A, this section) in a drop-dispensing bottle.

4. Germicidal solution (we use Econo-Quat, Unit Chemical Corporation, Los Angeles, CA 90066, at a concentration of 0.8 percent vol/vol in water).

5. Graph paper, logarithmic logistic ruling, Berkson (Codex Book Co., Norwood, MA 02062, catal. No. 32.454).

6. A computer program for logistic regression analysis. We use (a) program LOCSAN by R. L. Giese, 1968, Purdue University, Lafayette, IN, modified by L. Stewart, 1962, of this Experiment Station (minimum logit chi-square analysis of single bioassays; based on Berkson 1953), or (b) program LOMALI by D. A. Pierce and N. Srivastava, 1979, Oregon State University, Corvallis, OR (maximum-likelihood logistic regression analysis of single bioassays, or of multiple assays with comparison of slopes and of LD_{50} or LC_{50} values, after parallel-line fit; based on Cox 1970 and Finney 1977).

7. Microscope.

B. Procedure

1. Forty-eight h after inoculation: carefully examine each cup and discard those where no feeding has occurred or where feeding is barely evident or submarginal by comparison with the rest of the group. Record the number of feeding larvae in column "Group size at 48 h" on the infectivity test chart. (Based on our experience with strain FS-01, the mean rate of rejection computed over one year is 3.2 percent, or just one larva per group of 30 larvae. The number of larvae that fail to feed is not evenly distributed among the groups, however, and the "Group size at 48 h" can be as low as 26, in some rare instances.)

2. From day 3 to day 14 postinoculation, inclusive: examine each tray (daily or every other day, at the same time) and remove each cup containing a dead larva; place these cups in a tray, date, and refrigerate until diagnosis can be performed; or proceed directly to step 3.

3. Examine each larva macroscopically; if the integument is intact, probe with a toothpick or blood lancet; usually, the diagnosis (nucleopolyhedrosis) is readily apparent by macroscopic examination of the dead larva. Sometimes, microscopic examination of larval tissues may be necessary to confirm a diagnosis, however; use wet-mount preparations of tissue in buffered saline, to include epidermis, fat body, and tracheae.

4. Record the number of dead larvae (positive or negative for nucleopolyhedrosis) on the third row of each group section, on the infectivity test chart (fig. 8); the number of newly formed pupae may also be recorded in row "P" of the chart.

5. Save the caps: decontaminate by immersion in germicidal solution, wash, rinse, dry, and sterilize for repeated use. Autoclave and discard the cups with the diet and larval remains.

Group No.	Date treated	Inoculum PIB/Cup	Group size at 48 h	3-5	6	7	8	9	10	11	12	13	14	L
								Dead larvae (days postinoculation)						
5	DEC - 3 1980	75	29	-	$^{12}/_{9}$		$^{12}/_{11}$		$^{12}/_{13}$		$^{12}/_{15}$		$^{12}/_{17}$	
				P →	0	0	0	0	6	1	1	2	0	12
				0	0	0	1	4	$\frac{1\,neg}{5}$	2	$\frac{1\,neg}{2}$	1	0	-

P = pupated   L = live at completion

DEC - 5 1980	DEC - 6 1980 MEM	DEC - 7 1980	DEC - 8 1980	DEC - 9 1980	DEC 1 0 1980

Figure 8.—Completed group section of a record chart; note that two larvae (on days 10 and 12 postinoculation) were negative for nucleopolyhedrosis.

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6. At the end of the 14-d observation period, tabulate the data as in table 2. Only larvae that died of nucleopolyhedrosis (positive diagnosis) are used for data analysis (larvae that died of other causes are subtracted from the 48-h group size).

7_a. If the data are from a range-finding assay (see II, B_1, this section) and the mortality rates at two of the four dose levels fall between 10 and 90 percent, draw a line through the two points, on Berkson's logarithmic logistic ruling graph paper; the LC_{50} can be read on the X scale as the value corresponding to logit 0 (50-percent mortality) on the line. If only one mortality rate falls between 10 and 90 percent, draw a line with a slope of 2.5 through such a point; read the LC_{50} as explained above.

7_b. If the data are from a narrow-range assay (see II, B_2, this section) process the data by means of a logit-analysis computer program; enter the number of dose levels (4), the number of replications per dose level (2), and the data from columns b, c, and d (table 2). Most printouts list the LC_{50}, its 95-percent confidence limits, and the slope of the regression line; usually, the data are tested for nonlinearity and for heterogeneity of response between replications.

Table 2—Summary of bioassay data: an example

Group number (a)	Dose (PIB per cup) (b)	Number of test larvae[1] (c)	Number of dead larvae (nucleo- polyhedrosis) (d)	Mortality rate (percent) (e)
1	control	29	0	0
2	25	30	7	23.3
3	25	29	8	27.6
4	75	30	18	60.0
5	75	27	15	55.6
•	•	•	•	•
•	•	•	•	•
•	•	•	•	•
•	•	•	•	•

[1]48-h group size less larvae that died of causes other than nucleopolyhedrosis (compare with Group 5, fig. 8).

Results: An Example

A peroral bioassay procedure is a prerequisite for comparing the activity of *Baculovirus* isolates as inclusion-body suspensions. The following example illustrates a particular use of our procedure during the course of a screening program for viruses pathogenic for *C. occidentalis*.

We assayed, by the methods described, three multicapsid NPV isolates: CFU-MM[4], from *C. fumiferana*, CMU-MM, from *C. murinana*, and COC-MM, from *C. occidentalis* (see Appendix). Our aim was to compare the virulence of these isolates for the western spruce budworm. As a basis for comparison, we used the polyhedron-to-bioactivity ratio (PIB/LC_{50}). This ratio, combined with other characteristics of the pathogens' mode of action (such as the slope of the dose-mortality regression line and the mean survival time), can be used as one of the criteria for selection of a particular virus for production and field application.

The results of our bioassays, analyzed by program LOMALI, are presented in table 3. Methods for preparation of small quantities (0.5 g to 2 g) of PIB for these bioassays and for biochemical studies are described in the Appendix.

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[4]In this report we use the viral codes as proposed by Goodwin and Martignoni (1977): MM indicates *Baculovirus* morphotype with multicapsid virions and multiple (subgroup A) embedding.

Table 3—Activity titration of three *Baculovirus* isolates: logistic regression analysis (program LOMALI) and comparison of three assays

Virus	LC_{50} and 95-percent confidence limits, PIB/cup	Significance[1]	Precision[2]	Slope *b*	Significance[3]	Activity index[4]
COC-MM	54.83 (41.68 - 69.63)	a	1.67	3.32	d	100.0
CFU-MM	104.30 (74.06 - 144.40)	b	1.95	2.14	e	52.6
CMU-MM	472.30 (345.44 - 632.06)	c	1.83	2.41	de	11.6

[1]Values followed by different letters are significantly different (P is smaller than 0.001). Comparison after parallel-line fit.
[2]Confidence limit ratio (upper limit/lower limit).
[3]Values followed by different letters are significantly different (P is smaller than 0.05).
[4]Activity index computed according to Sun (1950). In this table, COC-MM is the standard.

Checklist of Items Needed for One Bioassay

Bags, plastic, 20- X 30- X 55-cm
Bottle, drop-dispensing, for saline
Brushes, No. 00, sterile (4)
Bulb assembly, rubber and glass, for
    capillary tubes
Caps, perforated, for analyzer cups,
    sterile (270)
Computer program for logistic regression
    analysis
Counter, one-unit
Counting chamber, Bright-line
Cover glasses (box)
Cups, analyzer, 2-ml, sterile (270)
Diet, modified McMorran (350 ml)
Dishes, petri, plastic, 60- X 15-mm,
    sterile (9)
Filler, rubber, for bulb pipetting
Germicidal solution
Graph paper, logarithmic logistic ruling
Hood, sterile
Hot plate, for water bath
Incubator, 25°C
Incubator, 30°C
Labels, adhesive (12)
Lancets, blood, sterile (box)
Larvae, fourth instars (270)
Marker, felt, fine-tip, permanent ink
Microscope (preferably phase-contrast)
Needle, hypodermic, 13G X 50-mm,
    sterile
Paper tissues, sterile (box)
Pipette tips, for 25-μl pipettor, sterile (16)
Pipettes, 1-ml, disposable, sterile (12)
Pipettes, 5-ml, disposable, sterile (12)
Pipettor, tip-ejector, 25-μl
Racks, Lucite, for trays (2)
Record charts, infectivity test (pages 1
    and 2)
Refrigerator
Saline, buffered, sterile (100 ml)
Slides, microscope (box)
Sterilizer, ultraviolet
Stirrer, magnetic
Stirring bars, magnetic, micro, sterile (8)
Syringe, Luer-Lok, 10-ml, disposable,
    sterile
Thermometer, 60°C
Toothpicks, sterile (box)
Trays, polycarbonate, for analyzer
    cups (9)
Tubes, capillary, 1.5- X 75-mm (12)
Vials, snap-cap, 16-ml, with caps,
    sterile (8)
Virus, stock suspension
Water bath

Acknowledgments

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Propagation and Processing of Experimental Quantities of *Baculovirus* in Larvae of *Choristoneura occidentalis*

Virus isolates.—The multicapsid NPV of the spruce budworm, *C. fumiferana* (CFU-MM)[4], was obtained in 1979 from J. C. Cunningham, Sault Ste. Marie, Ontario, Canada. For references to this virus see Arif and Brown (1975) and Cunningham (1978).

The multicapsid NPV of the fir budworm (fir shoot roller), *C. murinana* (CMU-MM), was a gift from J. Huber, Darmstadt, German Federal Republic. For references to the pathology and epizootiology of this virus see Krieg (1961) and Krieg and Huger (1969).

We recently isolated the multicapsid NPV of the western spruce budworm, *C. occidentalis* (COC-MM), from larvae of a wild population, collected in July 1979 at White Sulphur Springs, Montana. The larvae were obtained from G. P. Markin, Davis, California (C. G. Thompson, Forestry Sciences Laboratory, Corvallis, Oregon 97331, personal communication).

Virus propagation.—Three virus isolates were propagated in larvae of the diapausing colony of *C. occidentalis* strain FS-01. The insect-rearing procedures and diet formulation were essentially as described by Robertson (1979). We inoculated fifth, and occasionally sixth, instars by a diet-block treatment technique. We used an average of 500 larvae per virus isolate.

Blocks of solid diet of uniform size (0.6 cm^3) were dried at room temperature for 2 h, dipped into a suspension of PIB for 30 s, and dried at room temperature for 1 h. The mean PIB count per diet block was computed from the PIB count and rate of depletion of the suspension. Each block, with at least 100 LC_{50} units of PIB, was presented to 10 larvae, in a 60- X 15-mm plastic petri dish. The larvae were incubated at 30°C. After 48 h, they were fed a block (2 cm^3) of fresh diet (without virus). Beginning with the 6th day postinoculation, dead larvae were collected aseptically in plastic bottles and frozen. Surviving larvae were discarded on the 12th day. Only one virus isolate was propagated and processed at a time.

Virus processing.—Each of the three separate lots of frozen larvae was thawed, weighed, and homogenized in a blender for 8 min, at high speed, with addition of sterile ice-cold 0.02 M BES [N, N-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid] with 7.5 ppm DOSS (dioctyl sulfosuccinate, sodium), pH 7.15. The homogenized tissue suspension was passed through three stainless steel sieves (Tyler standard screen scale No. 100, 270, and 400) and the screened material was sedimented in a GSA Sorvall rotor (250-ml bottles) at 1020 g and 4°C for 30 min. The sediment was suspended in 48-percent (w/w) sucrose solution in BES-DOSS buffer (1.219 g/cm^3 at 20°C) and centrifuged in a GSA rotor at 27,300 g and 20°C for 60 min. If a microscopic examination of the sediment revealed the presence of many extraneous particles, this step was repeated in 48- or 50-percent (1.230 g/cm^3) sucrose solution. The sediment (fairly clean PIB) was then suspended and rinsed twice in BES-DOSS and once in BES-DOSS with 0.9-percent NaCl (centrifugation in a SS-34 Sorvall rotor at 755 g and 4°C for 15 min each time). The PIB were resuspended in sterile BES-DOSS-NaCl solution and absence of sucrose was checked with an Abbe 3-L refractometer. The final PIB suspensions were stored in 5-or 10-ml serum bottles, at 4°C. PIB counts and certain other quality control procedures were performed as described by Martignoni (1978).

Virus yield.—The PIB yield per larva and per gram of tissue, as well as the mean survival time (ST) of each production group are presented in table 4.

Table 4—Virus propagation in larvae of *Choristoneura occidentalis*: weight of production larvae, survival time (ST), and inclusion-body yield

Virus code	CFU-MM (No. 78)	CMU-MM (No. 77)	COC-MM (No. 81)
Natural host	*Choristoneura fumiferana* (Clemens)	*Choristoneura murinana* (Hübner)	*Choristoneura occidentalis* Freeman
Origin of inoculum	Forest Pest Management Institute, Sault Ste. Marie, Ontario, Canada (1978)	Institut für Biologische Schädlingsbekämpfung, Darmstadt, German Federal Republic (1978)	Forestry Sciences Laboratory, Corvallis, Oregon, United States (1979)
Mean weight of dead production larvae	82.9 mg	67.5 mg	43.8 mg
Yield after processing:			
Per larva	20.5×10^7 PIB	29.4×10^7 PIB	8.8×10^7 PIB
Per gram tissue	2.5×10^9 PIB	4.3×10^9 PIB	2.0×10^9 PIB
Mean ST at 30°C	8.8 days	9.8 days	7.6 days
Standard deviation	± 0.96	± 1.18	± 0.78

The relative virulence of entomopathogenic viruses and the potency of virus
preparations for control of destructive insects can be estimated reliably only by
means of biological assay in the target species. A simple, yet sensitive peroral
bioassay procedure is described for preparations of nucleopolyhedrosis viruses
pathogenic for the western spruce budworm, *Choristoneura occidentalis* Freeman.
This procedure has been used to compare the activity of *Baculovirus* isolates from
several conifer-feeding species of *Choristoneura*.